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14. ABSTRACT

During the current funding period, we have made clear progress at the University of Colorado site and with the other teams at Oregon Health and Science University and City of Hope. The major objective of this project is to develop novel strategies to enhance the protective effects of anti-tumor T cells *in vivo* in breast cancer (bc) patients based on the hypothesis that partially protective anti-tumor T cells exist within in most bc patients. This year we focused on which T cells should be used for epitope identification in bc patients. We started to develop an assay in which we sequence the alpha and beta chain of single T cells in one PCR reaction. We will use this protocol from T cells that are positive in an IFN-γ+ CD107a+ capture assay. This year we determined that culturing T cells in vitro results in significant skewing of the T cell repertoire. Thus, the sequence of the TCR of T cell clones expanded in culture must be compared with those isolated ex vivo. Finally we determined that T cells frequently present in the tumor and lymph node, but not frequently in the blood, should be analyzed. We are almost positioned to validate antigens and develop antigenic peptides recognized by T cells from bc patients.

15. SUBJECT TERMS
Breast cancer, tumor antigens, T cell receptor, cancer vaccine

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INTRODUCTION:

Two years into the Multi-Team Project ENHANCING THE BREADTH AND EFFICACY OF THERAPEUTIC VACCINES FOR BREAST CANCER (months 13 to 24), we have made clear progress at the University of Colorado site and with the other teams at Oregon Health and Science University and City of Hope. The major objective of this project is to develop novel strategies to enhance the protective effects of anti-tumor T cells *in vivo* in breast cancer patients. This is based on the hypothesis that partially protective anti-tumor T cells exist within most breast cancer patients. This year we focused on which T cells should be used to screen peptide libraries, so we can identify the epitopes recognized by T cells in breast cancer patients and mimotopes that activate them. We started to develop an assay in which we sequence the alpha and beta chain of single T cells in one PCR reaction. We will use this protocol for T cells that are positive in an IFN-γ+ CD107a+ capture assay. This year we determined that culturing T cells *in vitro* results in significant skewing of the T cell repertoire. Thus, the sequence of the TCR of T cell clones that are expanded in culture must be compared with those isolated *ex vivo* from the tumor. Finally we determined that the important T cells to analyze are frequently present in the tumor and LN, but not frequently in the blood.

The teams are working well together: we meet regularly on monthly conference call (organized by the OHSU group) and in person bi-annually. Since our last report, we met 2-26-13 at the City of Hope and 9-9-13 in Denver. In between formal meetings, the teams post results on a Google website (hosted by the OHSU group) and coordinate ongoing activities by phone and email. The necessary compliance issues are in place.

BODY:

The Tasks from the Statement of Work (revised 1-1-12) are in bold. Efforts toward these Tasks are in regular font.

Task 1. Generate reagents and identify conditions for experiments to follow: months 1- 40, Lee, Slansky, and Spellman

Reagents and experimental conditions have been created, acquired, and determined toward a number of different tasks. They are discussed within the appropriate tasks below.

Task 2. Enroll 100 patients with all major breast cancer subtypes from City of Hope and University of Colorado.

2a. Identify subjects with the appropriate BC subtypes

2b. Obtain patient consent and procure samples

To date, patient samples have been acquired to optimize our studies through Dr. Virginia Borges at the UC Anschutz Medical Campus (AMC) and Dr. Peter Lee at the City of Hope (Table 1). The teams agreed for the Slansky lab to use these specimens to develop and validate protocols. The samples were acquired under an IRB-approved protocol with written informed consent from each patient. None of the patients were previously treated with adjuvant therapy, i.e. radiation or chemotherapy. In the last 2 years, we obtained 16 fresh tumor specimens, 16 bloods and 1 tumor draining lymph node (TDLN) from the University of Colorado. We obtained 4 samples of isolated CD8+ and CD4+ T cells from fresh tumors and lymph nodes from the City of Hope this year. A summary of the patients is provided in Table 1.

Patient*	Subtype	HLA type	CD8 yield	CD4 yield	Sample use of CD8 TILs**
UC0147	Basal-like	HLA-A*02:01 HLA-DR*13:03	117,750	92,250	Used to analyze function of TIL and troubleshoot sequencing
UC0152	Luminal A	Not done	Not done	Not done	Tissue too small, not used
UC0157	Basal-like	HLA-A*02:01 HLA-DR*11:01	<6,000	<6,000	Used to troubleshoot sequencing
UC0197	Luminal B	HLA-A*02:01 HLA-DR*13:01	<4,000	<4,000	Sequencing
UC0198	Luminal B	HLA-A23 HLA-DR*07:01	68,000	24,000	TIL RNA stored for future use
UC0200	Luminal A	HLA-A*02:01 HLA-DR*01:01	T:30,000 LN: 37,500	T:15,000 LN: 62,500	Sequencing, TDLN included
UC0202	Luminal A	HLA-A*01:01 HLA-DR*07:01	<4,000	<4,000	TIL RNA stored for future use
UC0205	Luminal A	HLA-A*11:02 HLA-DR*09:01	<6,000	<6,000	TIL RNA stored for future use
UC0211	Luminal A	HLA-A*02:01 HLA-DR*04:01	16,000	Not done	Sequencing
UC0213	Luminal A	HLA-A*24:02 HLA-DR*01:01	Not done	Not done	Tissue had too much lipid, no selection
UC0217	Basal-like	HLA-A*29:02 HLA-DR*01:01	12,000	6,000	TIL RNA stored for future use
UC0221	Basal-like	HLA-03*01 HLA-DR*07:01	100,000	94,000	TIL RNA stored for future use
UC0238	Luminal B	HLA-01*01 HLA-DR*03:01	Not done	Not done	Used to trouble shoot IFN-γ capture assay
UC0255	Unknown	Pre-screen: HLA-A2+	Not known	Not known	Used to troubleshoot sequencing
UC0256	Basal-like	HLA-A*02:01 HLA-DR*13:04	256,000	76,000	Sequencing
UC0277	Basal-like	Pre-screen: HLA-A2+	Not done	Not done	Used to troubleshoot sorting specific populations for single cell PCR

*Other patients were consented, but we did not obtain tissues

**RNA from CD4 TILs is being store for future use

Patient	Subtype	HLA type	CD8 received	CD4 received	Sample use
C	Luminal A	HLA-02*01 HLA-DR*12:02	T: 10,000 LN: 10,000	T: 10,000 LN: 10,000	Sequencing comparison of *ex vivo* and *in vitro* expanded TIL
D	Luminal A	HLA-02*01 HLA-DR*09:01	T: 10,000 LN: 10,000	T: 10,000 LN: 10,000	Sequencing comparison of *ex vivo* and *in vitro* expanded TIL
E	Luminal A	HLA-02*01 HLA-DR*01:01	T: 10,000 LN: 10,000	T: 10,000 LN: 10,000	Sequencing comparison of *ex vivo* and *in vitro* expanded TIL
F	Luminal A	Pre-screen: HLA-A2+	NA	NA	Purity of the CD8 T cells was suboptimal, sample was unused.

*Table 1. Breast cancer patient samples from the UC AMC protocol (top) and selected samples from the City of Hope protocol (bottom). HLA-A*02:01 samples are underlined.*

Task 3. Process patient samples (blood, TDLNs, tumor): months 1-38, Lee and Spellman

3a. HLA typing of PBMC
3b. EBV transformation to generate B-LCL
3c. Disrupt and digest tumor and TDLN samples into single cell suspensions
3d. Isolate T cells by FACS sorting
3e. Expand T cells *ex vivo*

3a. We collect peripheral blood from patients at the time of patient consent (1-2 weeks prior to surgery). After Ficoll separation of the lymphocytes, we immediately check the patients' HLA serotype by flow cytometry. We use a mouse anti-human HLA-A2 antibody clone (BB7.2) to determine if the patient is HLA-A2+ (Figure 1, 1). This screen helps us decipher which experiments to pursue with each patient sample. HLA-A2 is the most common HLA allele in Northern Asia and North America populations; the allele-specific reagents we have currently developed in the lab are for HLA-A2+ patients. We also isolate genomic DNA and RNA by TRIzol extraction from $1x10^6$ PBMCs and send it to the City of Hope HLA typing lab, originally recommended by the Lee Lab, for high-resolution genotyping of the sample. The HLA in the HLA-peptide library that we have developed (discussed in Task 6) is the HLA-A*02:01 allele.

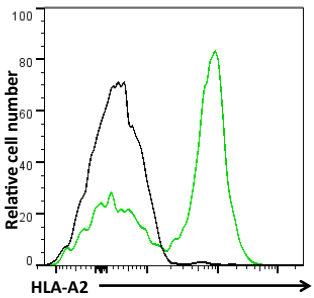


Figure 1. Staining with HLA-A2 antibody. PBMCS from patients were surface stained with the BB7.2 antibody to quickly determine if the samples should be genotyped at the City of Hope HLA typing lab. Black = HLA-A2- sample, Green = HLA-A2+ sample

3b. EBV transformation to generate B-LCL. (Lee lab)

3c. Once tumor and TDLN are collected, the tumor tissue are processed and used according to Figure 2A (2). TDLN are processed similarly to tumor, although they do not require a digestion with the liberase enzyme, unless there are a lot of tumor cells in the lymph node. To date, we obtained one TDLN

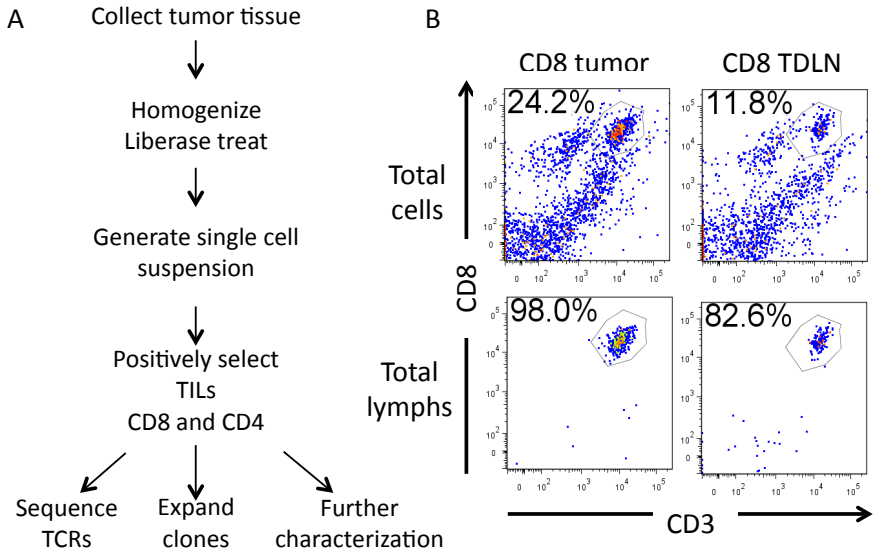


Figure 2. Tissue processing and TIL selection. (A) Flowchart of processing of tumor samples. (B) Successful enrichment of TILs from breast cancer patients. There is significant patient-to-patient variation in the yield of T cells obtained from each sample. In addition, tumor development, tumor size, and sample size contribute to the final yield. We have obtained 5,000-250,000 T cells per sample with a post selection purity compared to all cells in the tumor sample of 10-50% (top panel) and > 80% of other lymphocytes (lower panel).

(UC0200). Within two hours of surgery, specimens are retrieved and processed. We are focusing on the CD8 T cells, although we are storing CD4 T cells for future experiments. After the positive selection, the overall purity of the samples was between 10-50% (Figure 2B, top panel). The lower purity is due to tumor cells being pulled through with the positive selection, not other lymphocyte contamination. The lower purity of the TDLN many be due to the method in which the sample was extracted before being processed in the lab. However, because we have only obtained one TDLN, we have not tested this yet. In fact, in analyzing just the purity of lymphocyte population, the purity is over 90% CD8 tumor infiltrating lymphocytes (TILs, Figure 2B, bottom panel). In this report, we discuss sequencing of the TCRs, expansion of the clones, and further characterization of the CD8 TILs.

3d. CD8 (TILs) are isolated using Dynal positive selection kits (Invitrogen) or FACSorting. To show that CD8 TILs from breast cancer can indeed be stimulated and to identify which T cells should be used for antigen discovery, we isolated CD8 TILs as indicated in Figure 1, and then determined their function after stimulation with antibodies to CD3 and CD28 or PMA/ionomycin (Figure 3A). Analysis of CD8 TIL stimulation showed significant proliferation and a robust amount of IFN-γ production when stimulated through the TCR (αCD3 and αCD28) for five days. This method of identification of these stimulated T cells requires fixing, or permeablizing, the T cells. Thus, we determined the conditions for a "capture assay" which can viably identify T cells, so they can be isolated by FACSorting and further used (Figure 3B).

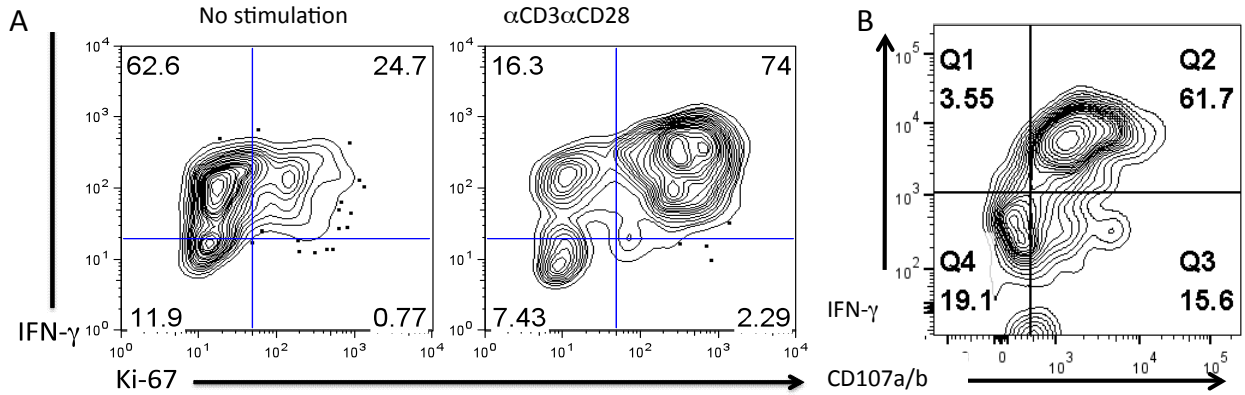


Figure 3. Some breast cancer CD8 TILs produce IFN-γ, proliferate, and express CD107a suggesting that they can be stimulated by a vaccine. (A) CD8 TILs were isolated as in Figure 1 and stimulated with antibodies to CD3 and CD28. After 5 days the T cells were stained with Ki-67, a marker of proliferation, and stained intracellularly for IFN-γ. Many of the cells that produce IFN-γ also proliferate in response to stimulus. (B) CD8 TILs were stimulated for 6 h in vitro with PMA and ionomycin. These mitogens bypasses the TCR signal and nonspecifically stimulate the cells to produce cytokines. IFN-γ production was determined using a capture assay and stained with a CD107 antibody. These T cells are viable, can be sorted by FACS, and used to determine antigen-specificity.

3e. Expand T cells *ex vivo*.

We received one set of expanded T cells lines from the City of Hope which are discussed in detail below. To determine if the populations of *ex vivo* T cells were similar to the populations following α-CD28/α-CD3 bead stimulation, i.e. to determine if skewing occurs during expansion, RNA from the CD8+ T cell

samples listed in Table 2 was extracted and the TCRs were sequenced. For each paired sample, approximately half the cells procured were used for the expansion.

Sample	Total α reads	# Unique CDR3α sequences	Total β reads	# Unique CDR3β sequences	# Input CD8cells
C1 LN	4,250,006	4,201	3,656,661	4,950	200,000
C1 LN Exp.	3,705,705	14,529	2,679,005	13,721	ND
D1 TIL	2,085,766	2,344	2,463,014	1,444	38,000
D1 TIL Exp.	4,405,298	13,691	1,827,522	10,674	ND
D1 LN	1,564,348	4,371	467,900	4,102	1,240,000
D1 LN Exp.	6,603,224	38,431	1,569,268	25,118	ND

Table 2. Samples assayed for population skewing. Unique CDR3 sequences include all CDR3 sequences which were present 2 or more times. The number of input cells for the expansion was not determined prior to TRIzol extraction.

Extracted T cell RNA was reverse transcribed and amplified using a primer set from iRepertoire Inc. which specifically amplifies either alpha or beta TCR RNA. Equimolar amounts of PCR amplicon for each sample was submitted to iRepertoire for sequencing and bioinformatic analysis. The results of the sequencing gave a range of total reads and numbers of unique CDR3s for each sample (Table 2). In each case, the number of unique CDR3s was higher in the expanded samples, likely due to the fact that single founder T cells in the unexpanded population, which would be difficult to detect from a total RNA pool, expanded to detectable numbers in the post-expansion population.

To assess change in T cell populations, we first analyzed the change in overall V usage between pre- and post-expansion samples. For each sample, the usage frequency of a specific TCR V region in the pre-expansion population was subtracted from the frequency in the post-expansion population to give an overall change in frequency (Figure 4). Thus, a positive change indicates an increase in the post-expansion population and a negative change indicates the V region frequency was higher in the pre-expansion population. Among the TRAVs sequenced, there was a maximal increase in frequency of TRAV4 (+6.8%) and a maximal decrease of TRAV25 (-6.1%). The maximal change within the TRBVs occurred with TRBV27 (+11.3% and -14.8%). Samples taken from the same patient (D1) tended to change similarly, however there were occasions where the V gene usage was different. Comparing patient C1 to D1 there was no correlation with respect to frequency change in V gene usage. For TRAV gene usage, 35.5% of the values are outside the 95% CI (<+0.171% and -0.15%). For the TRBV usage, 50% of values were outside the 95% CI (<+0.26% and -0.37%).

The human αβ TCR consists of a V region paired with a J region (and a D region in the beta chain, of which there are only two). For the alpha TCR, there are 45 unique V regions and 50 unique J regions, giving a total VJ diversity of 2,250 possible V-J combinations. The beta TCR has 47 V regions and 13 J regions, yielding 611 possible V-J combinations. As plotting this many combinations on a linear graph would be overly cumbersome, we plotted the relative rank of specific VJ combinations based on the frequency at which the combination occurred (Table 3). We used rank so that each VJ combination is on the same scale, making direct comparisons easier and standardized. Rank is defined as the total number

of sequence positions +1 (7 positions in Table 3, top) minus the sequence position of interest, divided by the total number of sequence positions. This allows the scale of ranks to be from 0 to 1, as opposed to looking at different scales when looking at frequency.

Figure 5 shows the change in VJ usage in both the alpha (A-C) and beta (D-F) chains, where each line represents an individual VJ combination connecting its positional rank in the pre- and post-expansion populations. Using the frequency at which a particular VJ combination was utilized as the weight, a trend line was created (black lines) to illustrate the overall change in VJ usage from pre-expansion to post. The steeper the slope, the more infrequent VJ combinations in the pre-expansion population dominate in the post-expansion population (also evidenced by the large number of lines starting at 0 in the pre-expansion population to >0 in the post-expansion population). Within a specific sample, the trend in change of alpha and beta TCRs was of a similar magnitude.

We next wanted to address whether the overall length distribution of the CDR3 region changed during *ex vivo* expansion. For each sample the length of the CDR3 was calculated in nucleotide base pairs starting from the X of the V region and ending at the X of the J region. The lengths are plotted as the proportion of the total (Figure 6).

To more specifically address the fate of specific T cell clones, we analyzed the change in frequency of unique CDR3s for both alpha and beta (Figure 7). A unique CDR3 region was defined as the V-CDR3-J combination, since a CDR3 could have the same sequence as another with a disparate V and/or J. The change in frequency was determined by taking all unique pre-expansion CDR3s, which were sequenced, and determining the frequency in the post-expansion population. The pre-expansion frequency was then divided by the post expansion frequency to yield a fold decrease in frequency. Each unique CDR3 was plotted along the x-axis with a position conferred by the fold change. Sample C1 LN (Figures 7A,B), D1 LN (Figures 7C,D), and D1 Tumor (Figures 7E,F) each have clones which decrease by more than 100 fold. The percentage of clones that decrease by this amount ranges from 15-33% and the beta and alpha clones from the same sample decrease similarly. Clones which have a decrease of <1 are those which increased in the post-expansion population compared to the pre-expansion population. They are included in the percentages based on where they fall (i.e. a value of .01 would be a 100 fold increase in frequency). In most cases there were very few of these situations and the clone represented did not become a dominant clone in the post-expansion pool.

Summary of results for Task 3e: Although expansion of T cells *ex vivo* provides us with a greater number of cells for analysis, the population dynamics change such that highly frequent clones present in the pre-expansion population are lost in the post-expansion population. Additionally, some V regions and VJ combinations dominate the post-expansion population but are negligibly frequent in the pre-expansion population. While between 29-63% of clones differ by <10 fold between the populations, there is no guarantee that these are tumor specific clones; even though some clones are highly frequent in the pre-expansion population and remain so following expansion. Furthermore, it is well established that tumor resident T cells are often dysfunctional, and when removed from the tumor microenvironment do not proliferate and die. Because of the observed skewing we have decided to pursue direct *ex vivo* sequencing.

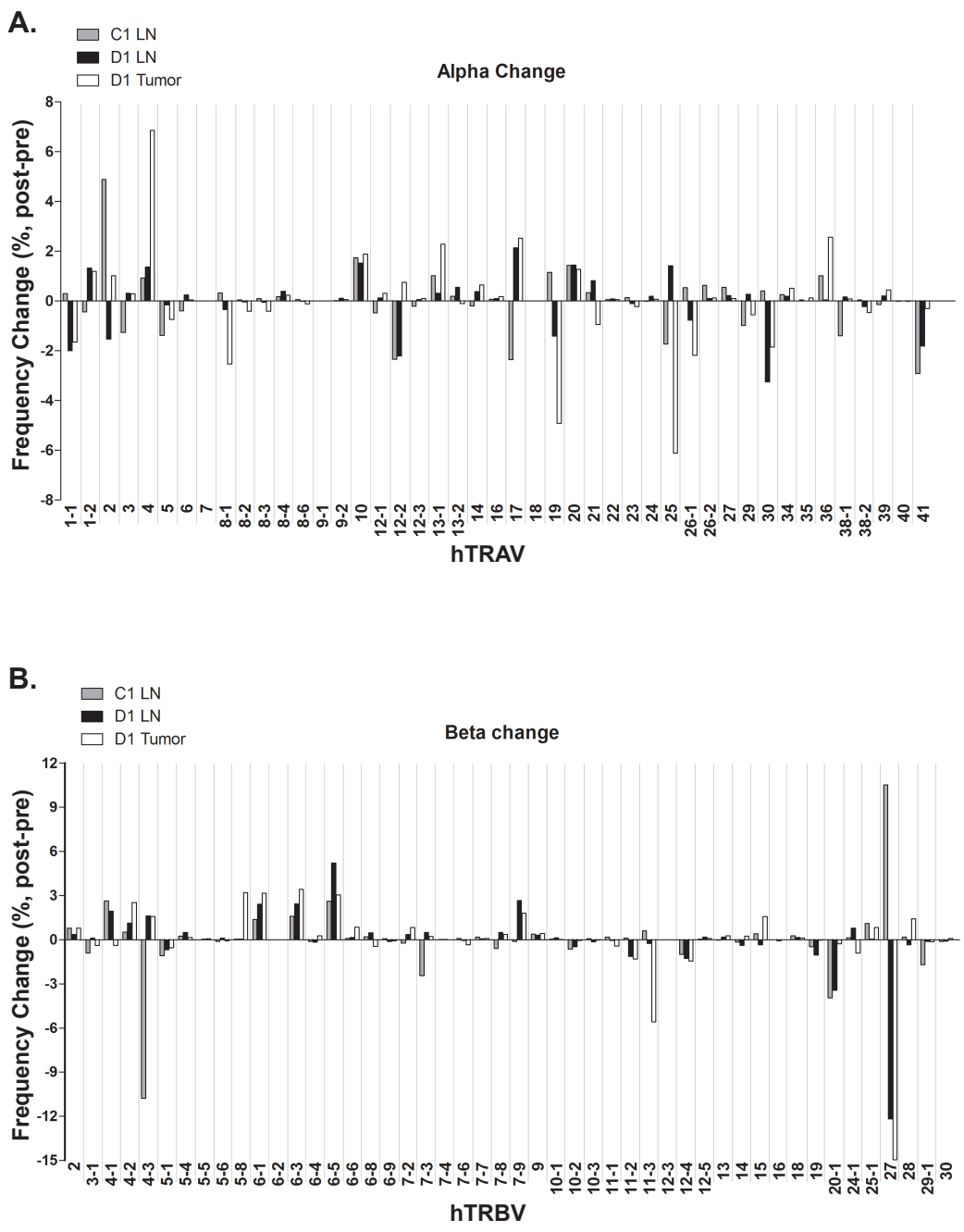


*Figure 4. The frequency of V usage changes during ex vivo expansion. The frequency of TCRAV (**A**) and TCRBV (**B**) usage changes similarly with respect to samples acquired from the same patient (D1 LN and Tumor). Differences in frequency between patients does not correlate. The specific pre-expansion TCR V regions were subtracted from the post-expansion TCR V regions for a change in frequency.*

A.

CDR3VJ identity	# sequences	Frequency	Position	Rank
ASVADRTGQETQY_hTRBV27_hTRBJ2-5	2500	33.784	1	1
ASSFSVNTEAF_hTRBV27_hTRBJ1-1	1500	20.270	2	0.857
ASSSSDRDTDTQY_hTRBV11-3_hTRBJ2-3	1200	16.2164	3	0.714
ASSLSSSSYEQY_hTRBV27_hTRBJ2-7	1000	13.514	4	0.571
ASSQGQSSYEQY_hTRBV4-1_hTRBJ2-7	500	6.757	5	0.429
SAREEGYEQY_hTRBV20-1_hTRBJ2-7	200	2.703	6	0.286
ASSLGQIYGYT_hTRBV6-3_hTRBJ1-2	200	2.703	6	0.286
ASSDRVGGYT_hTRBV27_hTRBJ1-2	150	2.027	7	0.143
SASGAYNEQF_hTRBV20-1_hTRBJ2-1	150	2.027	7	0.143

B.

CDR3VJ identity	# sequences	Frequency	Position	Rank
ASVADRTGQETQY_hTRBV27_hTRBJ2-5	2500	16.217	1	1
ASSFSVNTEAF_hTRBV27_hTRBJ1-1	2400	15.569	2	0.875
ASSQGQSSYEQY_hTRBV4-1_hTRBJ2-7	2300	14.920	3	0.75
ASSDRVGGYT_hTRBV27_hTRBJ1-2	1900	12.325	4	0.625
SAREEGYEQY_hTRBV20-1_hTRBJ2-7	1900	12.325	4	0.625
ASSLGQIYGYT_hTRBV6-3_hTRBJ1-2	1800	11.676	5	0.5
SASGAYNEQF_hTRBV20-1_hTRBJ2-1	1400	9.0815	6	0.375
ASSLSSSSYEQY_hTRBV27_hTRBJ2-7	800	5.189	7	0.25
ASSSSDRDTDTQY_hTRBV11-3_hTRBJ2-3	416	2.698	8	0.125

Table 3. Example of how ranks are determined. Specific sequences appear in the total population a certain number of times (column 2, # sequences). The frequency and the position in the list of TCRs can be used to determine rank (last column), allowing samples for which the frequencies maybe disparate and difficult to compare to be scaled equally from 0-1. One is the highest ranking clone (and therefore the most frequent) and 0 is non-existent. Rank is calculated by applying the following formula: ((Total # of Positions+1)-position of interest)/total # of positions (7 in A, 8 in B). In the example above, the frequency range is from 34%-2% in the pre-population (A) and 16%-3% in the post-population (B), despite the same two clones being #1 and #2. Ranking scales them similarly from 1-0 making comparison easier visually.

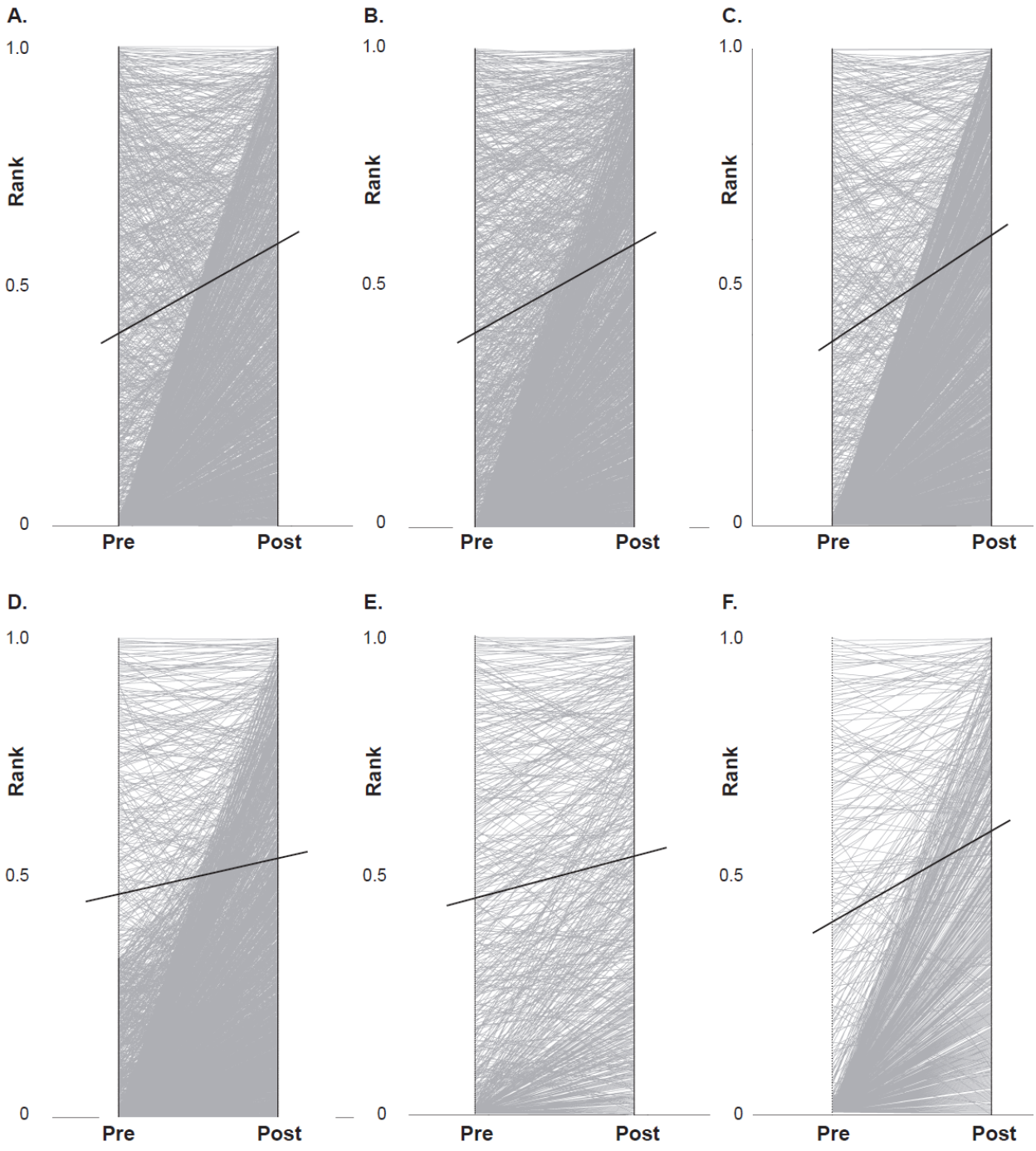


Figure 5. VJ usage changes following ex vivo *expansion. A) C1 LN alpha, B) D1 LN alpha, C) D1 Tumor alpha, D) C1 LN beta, E) D1 LN beta, F) D1 Tumor beta. Grey lines represent an individual VJ combination and connect the positional rank in the pre- and post-expansion population. The trend lines (black lines) are weighted averages of the slopes of each grey line, using the frequency of each VJ combination in the post-expansion population as the weight.*

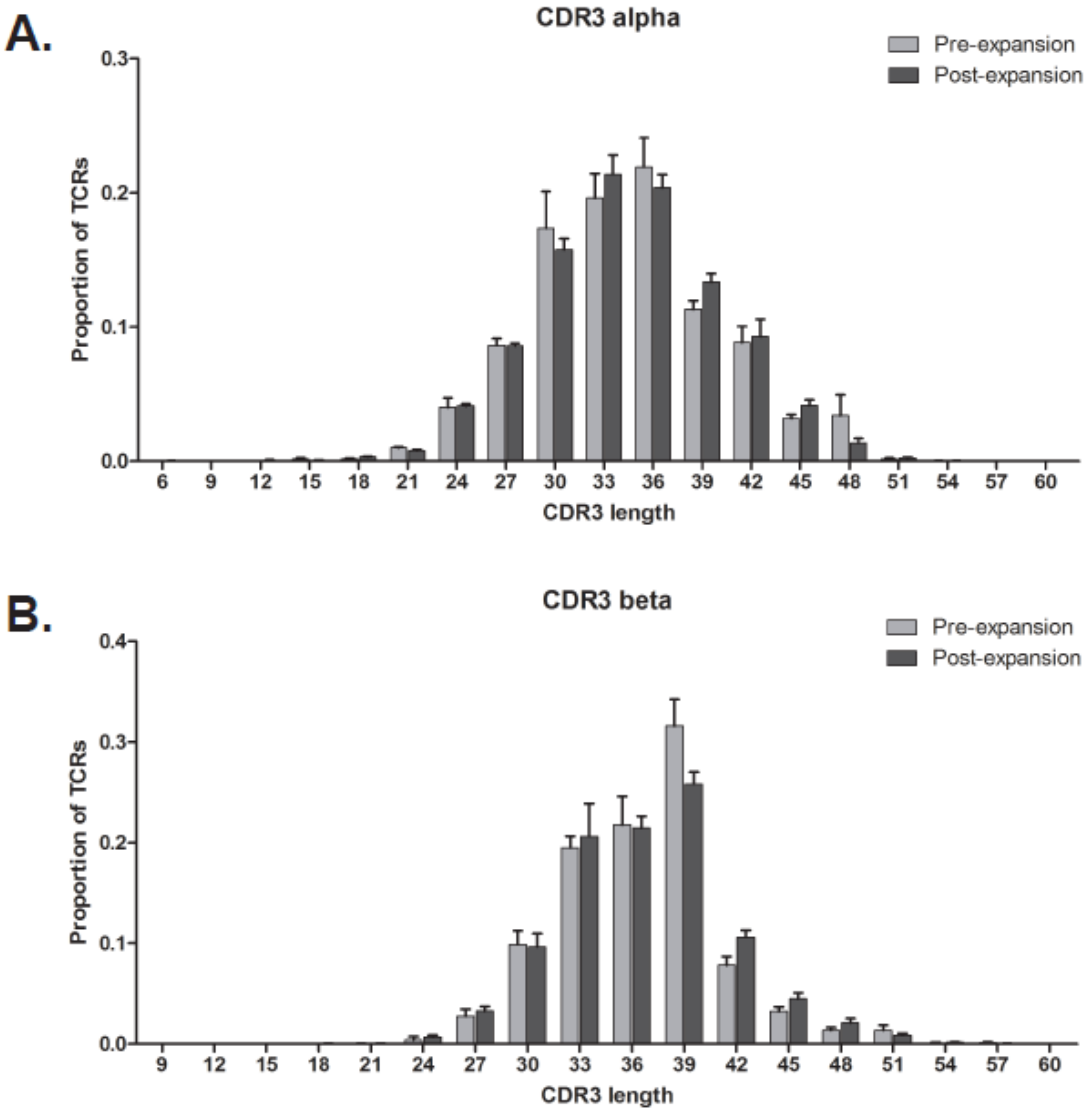


Figure 6. *CDR3 length distribution of unique CDR3VJ clones is not statistically different between the pre- and post-expansion populations. CDR3 length was determined for both alpha (A) and beta (B) unique TCR CDR3s and is displayed in nucleotide length. Lengths were measured from the nucleotides encoding the cysteine at the 3' end of the V gene and the phenylalanine at the 5' end of the J gene. Error bars represent the standard error of the mean.*

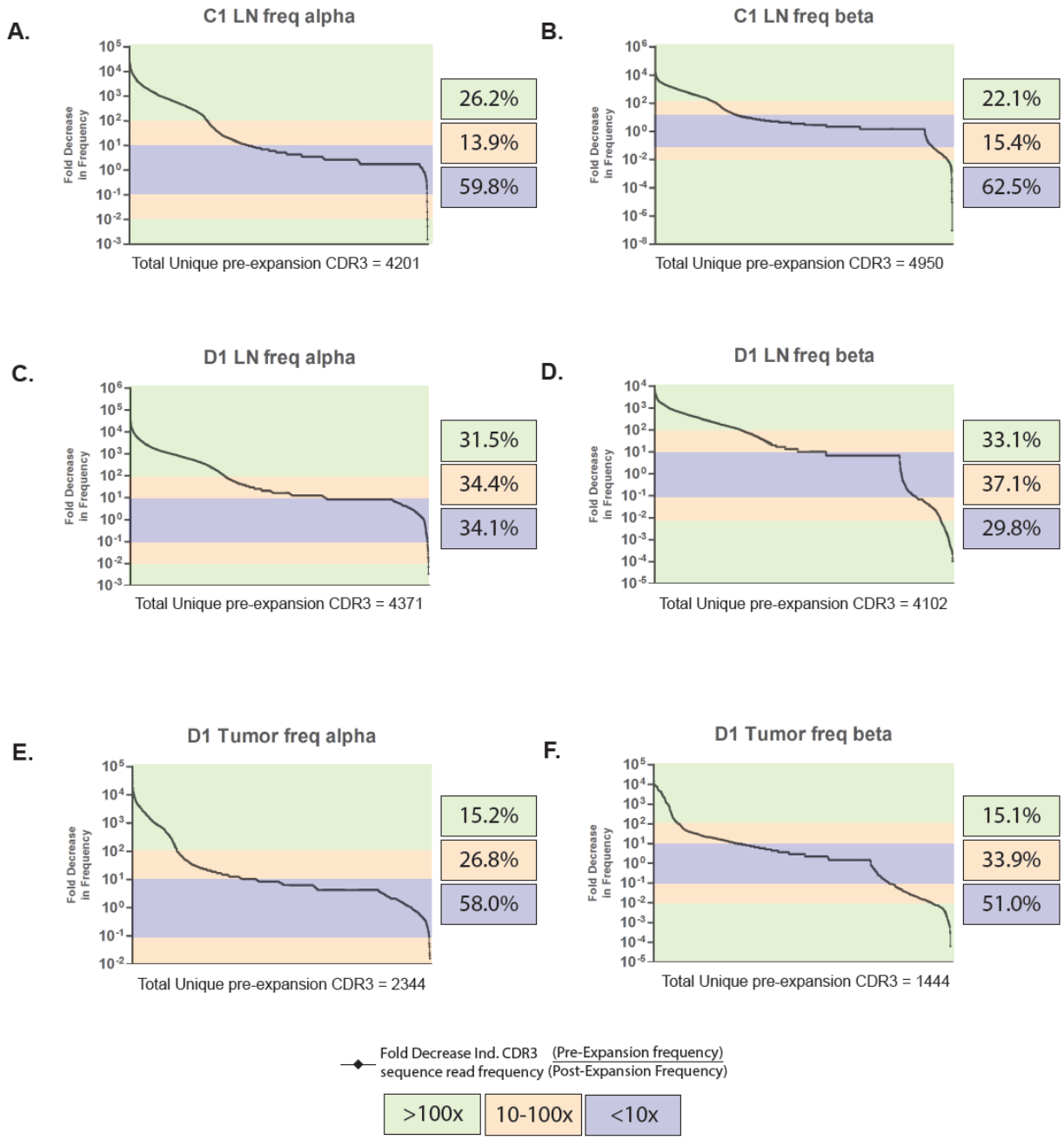


Figure 7. TCR alpha and beta CDR3-defined clones primarily decrease in frequency during ex vivo expansion. Unique CDR3VJs were analyzed for their decrease during ex vivo expansion by comparing the pre-expansion frequency to the post-expansion frequency. Clones were segregated into three groups consisting of >100-fold change (green), 10-100-fold change (salmon), and <10-fold change (blue). Increases in frequency are displayed as numbers less than 1 and are grouped according to fold increase below 1. Percentages next to the graphs indicate the total proportion of clones which fell into each group.

Because of the of the skewing that occurs in culture, when we receive samples, from either the City of Hope team or locally we will employ an emulsion-based RT-PCR method to isolate alpha/beta paired RNA from single cells. Results from this procedure suggest not only what alpha and beta TCRs are found together, they will also suggest the frequency of specific pairs found within a sample. In subsequent sections (Task 5) we provide evidence for comparing the blood, lymph node, and tumor TCRs to choose appropriate TCRs for further study. The protocol in Box 1 will be employed on the single cell scale as the conditions are determined.

Box 1. Protocol for emulsion-based RT-PCR (based on ref 3)

- Pellet and resuspend in 10 µl of 150 mM NaCl.
- Prepare a master mix of the Qaigen One-Step RT-PCR mix, 0.8 uM Calpha and Cbeta specific primers, and an oligo specific to an overlap sequence designed into the V region primers, 0.5 µM V region mixed primers (47 alpha, 45 beta primers), and 0.5 g/l BSA, 100 µl total volume.
- Stir a mixture of 2% ABIL EM 90, 0.05% Triton-X 100 in mineral oil (900 µl total volume), on ice at 1400 rpm for 2 min. Add the aqueous master mix dropwise over a period of 2 min while stirring. Stir an additional 5 min.
- Pipet 125 µl of emulsion into 8-tube PCR strip tubes and cycle 65^{o}C for 2 min, 50^{o}C for 30 min, 95^{o}C for 15 min, and 40 cycles of 94^{o}C for 45 s, 61^{o}C for 45 s, 72^{o}C for 50 s, followed by 72^{o}C for 10 min.
- Collect the PCR emulsion into two microcentrifuge tubes and spin @ 13,000 x *g* for 5 min. Remove the oil phase and extract 2x with 1 ml of water-saturated diethyl ether, 1x with water-saturated ethyl acetate, and 2x more with ether. Remove residual ether with a SpeedVac.
- Cleanup the PCR with a column and perform a nested PCR using Calpha and Cbeta primers and an excess of "blocking" oligos which have the same sequence as the step out oligo but contain 7 T nucleotides on either end as well as a 3' phosphate group.
- Sequence using Illumina MiSeq 2x150 bp paired end sequencing.

Task 4. Identify and isolate anti-tumor T cells from TDLNs and tumor samples: months 1-40, Lee, Slansky, and Spellman

4a. Generate tumor lysates pooled from BC cell lines of each major subtype (luminal, HER2+, basal)—Spellman

4b. Generate specific tumor antigen lysates from recombinant baculovirus-infected insect cells.

4c. Generate autologous tumor targets via B-LCL presenting tumor lysates

4d. identify and isolate anti-tumor CD8 T cells via CD107 mobilization, and anti-tumor CD4 T cells via CD154 mobilization

4a. Generate tumor lysates pooled from BC cell lines of each major subtype—Spellman

4b. Generate specific tumor antigen lysates from recombinant baculovirus-infected insect cells. We plan to screen T cells against a panel of known BC antigens prior to screening libraries. If the T cells react with these known antigens, the road to antigen and mimotope discovery will be greatly shortened. Toward this goal, we have stably transfected C1R:A2 cells (from Vic Engelhard at University of Virginia) with known breast cancer antigens HER2, NY-ESO-1, hTERT, CEACAM5, and MUC-1 (Figure 8). Evidence of tumor antigen expression is suggested by detection of GFP fluorescence and by specific antibody staining. In addition to these well-studied breast cancer antigens, we are also considering adding some differentially expressed genes from the Spellman team.

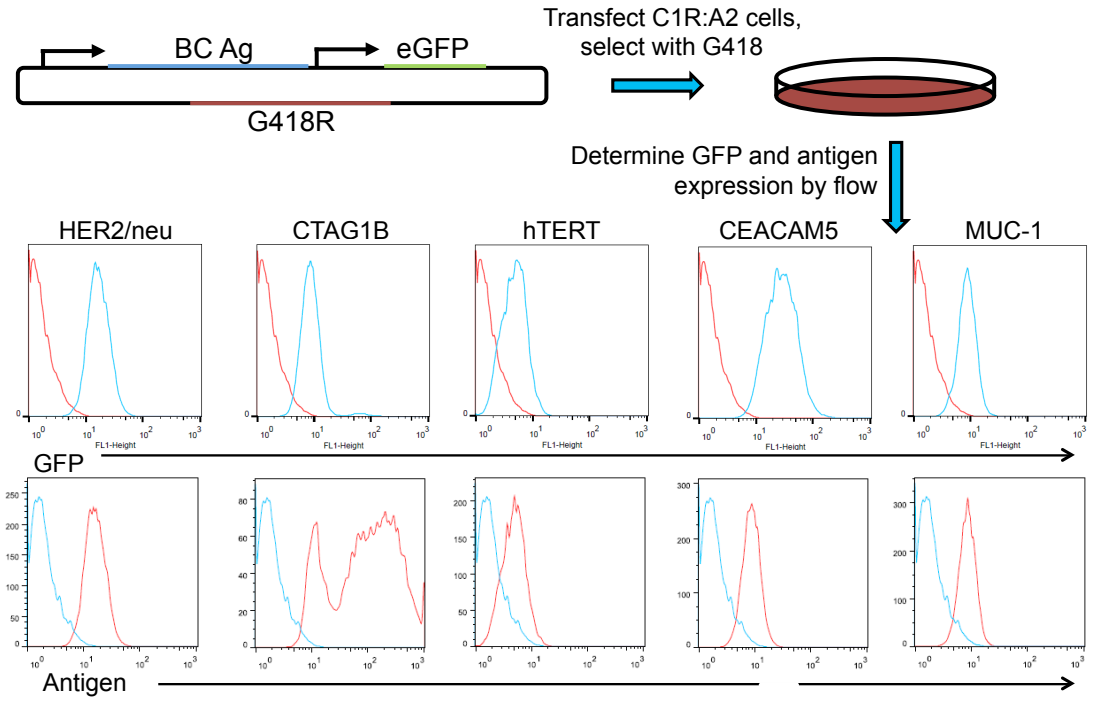


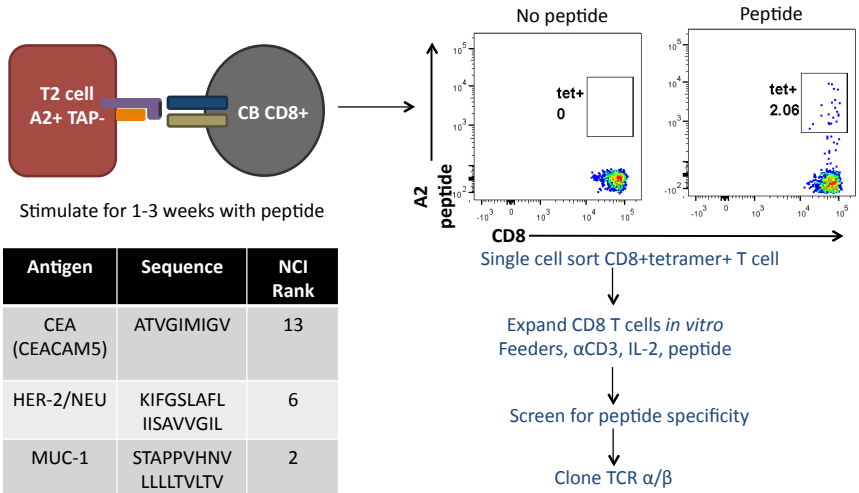
Figure 8. Generation of HLA-A2+ cell lines expressing specific breast cancer antigens. Human C1R cells transfected with HLA-A2 are an EBV transformed B cell lymphoblast line and will process antigen for presentation of A2-specific epitopes. Top row, GFP in blue. Bottom row, antigen in red.

To confirm that the specific HLA-A*0201-restricted tumor antigens are processed and presented by these cells, not just expressed, we are generating antigen-specific T cells. We are making these T cells by stimulating cord blood T cells in culture with known peptides of breast cancer antigens loaded onto T2 cells in the presence of IL-2 and CD3 antibody. Figure 9 shows how we are isolating the antigen-specific T cells. We will sub-clone the TCRs, insert them into hybridoma cell lines to make transfectomas, and use these to indicate antigen recognition on the C1R:A2-tumor antigen cell lines. This methodology will continue to be helpful when screening the peptide library as the total number of T cells may be limiting.

The generation of transfectomas and an example of the process are detailed in Figure 10. Briefly, RNA is extracted from the T cells. A unique anchor is ligated to the 5' end of all transcripts and the RNA reverse transcribed into cDNA. PCR is performed with either an alpha or beta TCR reverse primer that binds to a conserved sequence in the constant region, and a forward primer specific to the linker. The reverse primer has a restriction site tail to aid in cloning. The PCR product is digested and cloned into plasmids used in transducing a T cell hybridoma that has little to no natural TCR expression, but expresses human CD8. The plasmids also contain GFP downstream of an IRES used for cell sorting in combination with the

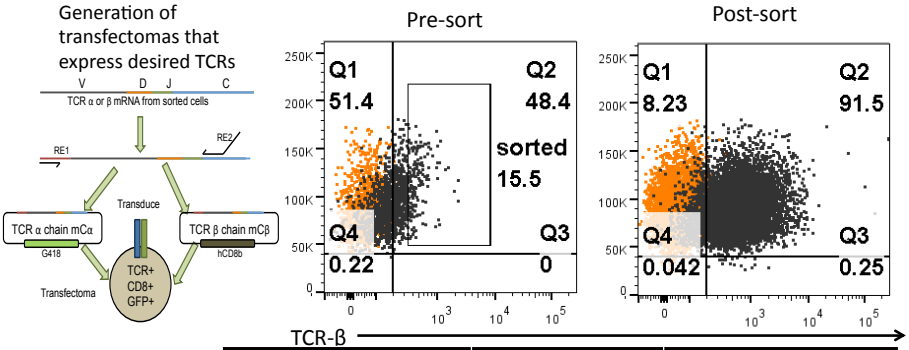
surface expression of the TCR molecule. TCR functionality can be assessed by binding to a potential target and measuring the release of IL-2 by ELISA.

In this example (Figure 10), the most frequently identified TCR α and β chains from patient UC0197 were synthesized and cloned into retroviral vectors. Ten days after transfection the cells that expressed TCR β chains were sorted and grown out. The next step with these transfectomas, along with the positive control breast tumor antigen-specific T cell trasfectomas, is to determine if they are activated by any of the C1R-A2 cell lines described in Figure 8.



Antigen	Sequence	NCI Rank
CEA (CEACAM5)	ATVGIMIGV	13
HER-2/NEU	KIFGSLAFL IISAVVGIL	6
MUC-1	STAPPVHNV LLLLTVLTV	2
NY-ESO-1	QLSLLMWIT	10
Telomerase	ILAKFLHWL	23

Figure 9. Generation of T cells specific to breast cancer antigens. CD8 T cells were positively selected from HLA-A2+ cord blood samples and stimulated with peptide-pulsed T2 cells at a 10:1 E:T ratio in the presence of IL-2 and αCD3. Cells were stimulated for 1-3 weeks and screened weekly for peptide specificity. Once the CD8 T cells demonstrated peptide specificity by tetramer staining, the cells were single cell sorted for clonal expansion. We plan to subclone the α/β TCRs for the generation of transfectomas. The table shows the sequence of the peptides used for generation of the clones and their ranking by Cheever et al. All of the antigens besides NY-ESO-1 were also identified by the OHSU team.



αβ pairs	α sequence	β sequence
Vα23Jα34, Vβ9Jβ2-7	CAASGGTDKLIF	CASSADRGDYEQYF
Vα13-2Jα20, Vβ9Jβ2-7	CAESPRPGGADGLTF	CASSADRGDYEQYF
Vα17Jα20, Vβ9Jβ2-7	CATVGSNDYKLSF	CASSADRGDYEQYF
Vα23Jα34, Vβ21-1Jβ2-6	CAASGGTDKLIF	CASSFRNRGSKSGANVLTF
Vα13-2Jα20, Vβ21-1Jβ2-6	CAESPRPGGADGLTF	CASSFRNRGSKSGANVLTF

Figure 10. Create a transfectoma using a specific αβ TCR pair. TCR mRNA was isolated from T cells from Patient UC0200, inserted into retroviral vectors, then transduced in the 5KC-based hybridomas. The TCR sequence was translated and expressed on the surface of the transfectoma cells (orange dots—untransduced cells, dark dots—transduced with TIL TCRs, ready for screening). If the TCR of these transfectomas bind to the cognate peptide, they will produce the cytokine IL-2.

Task 5. Generation and initial analysis of T cell clones: months 1-40, Lee, Slansky, and Spellman

5a. Expand isolated T cells as clones
5b. Confirm tumor reactivity and HLA restriction of each clone
5c. TCR sequencing of each clone
5d. Select 10 unique CD4 and 10 unique CD8 clones from each patient for further analysis

5a. See 3e above.

5c. Sequencing of TCRs from breast cancer patients. We have sequenced patient samples from both City of Hope and from University of Colorado including the bulk TCRs from the tumor, draining lymph node, and blood. Bulk TCR sequencing was done by extracting T cell RNA using TRIzol and performing RT-PCR using the iRepertoire primer sets introduced in Task 3. PCR amplicons were sent to iRepertoire for sequencing on the HiSeq2500 and the V, J, and CDR3 sequences were identified. The samples that were collected are summarized in Table 4 and the number of sequences acquired in Table 5.

The patients included in the analysis ranged in age from 32-79 years old and were all female. All are HLA-A2:01 and have invasive ductal carinomas comprised of luminal A (5), luminal B (1), or basal (1) subtypes. Molecularly, 6 patients were ER and PR positive, while only 2 were Her2+. There were no similarities in the HLA-DR types of the patients. For each patient the numbers of cells collected ranged from 20,000 up to 250,000 for a tumor sample and from 200,000 to 1.24 million for the lymph node samples. For the blood, a standard of 1 million CD8+ lymphocytes were collected. As with the expansion samples, the total numbers of reads as well as the number of unique CDR3s, although variable, were similar in all samples. In only a single case did the number of reads not exceed the number of input CD8 cells, although the diversity of unique CDR3 reads was similar to those where the number of reads exceeded the number of input cells.

We next determined if there was a preference for specific V region(s) within the TIL. Figure 11 shows the frequency of V usage for each patient sample according to where the cells were collected. V usage between patients, although variable for a given V alpha or beta, trended similarly and there were very few instances where a specific V region was elevated in all patients. Preferences for a specific V region within the TIL were determined by comparing the frequencies in the tumor to the blood and to the lymph node (Figure 12). Positive frequency differences represent V regions which were more prevalent in the tumor, whereas negative values indicate V regions more prevalent in either the lymph or the blood. Because T cells were collected from the primary tumor draining lymph node, we would expect the V usages to be relatively similar. Additionally, because the patients have no documented metastatic disease we would expect the blood T cell repertoire to be relatively different than the tumor repertoire. Indeed, for TRBV we observed very similar frequencies in the tumor and lymph node (Figure 12D) and more disparate frequencies in the tumor and blood (Figure 12B). For TRAV there is not as much correlation between frequencies in the tumor and lymph node (Figure 12C), which is mirrored in the tumor and blood (Figure 12A) and would be what we expect. Differences in V usage observed in Figures

11 and 12 were used to infer which CDR3s discussed in subsequent figures would be likely candidates for further analysis.

Rates of unique CDR3 sequences were tracked in the tumor and compared to the blood (Figure 13) or the lymph node (Figure 14). As mentioned, we expected the T cells from the tumor to be more like those in the draining lymph node than the blood.

Trend lines, which were calculated by taking the weighted averages of the slopes of each line, represent the overall similarity of the tumor to the other compartments. Similar to the V usage analyses, we would expect the trend lines to be flatter when comparing the tumor to the lymph nodes (Figure 14) due to the lymph node being the source of TIL. Alternatively, we would expect the trend in the tumor vs. the blood to be more steeply angled because there should not be many tumor specific T cells in the periphery. Furthermore, CDR3 sequences which are high in both the blood and tumor are more likely to be non-tumor specific T cells which happened to be migrating through the tumor at the time of resection. Tumor vs. blood comparison generally showed that highly frequent CDR3 sequences in the tumor are not present in the blood, with the most stark example of this being patient 211 (Figures 13C,G). When the tumor CDR3 sequences are then compared to their ranks in the lymph node, the trend line begins to flatten and indeed when we look at CDR3s on an individual basis, highly frequent clones from the tumor remain highly frequent in the lymph node (Figure 14). Used in tandem, we can select CDR3 sequences that we would predict to be tumor specific based on being highly frequent in the tumor, relatively frequent in the lymph node, and infrequent in the blood.

Because patient 211 is such a stark example of tumor disparity to blood, a table was created with the ranks and frequencies of individual CDR3s, which would be ideal candidates for further analysis (Table 6). Sequences were considered if the rank in the LN was above 0.75 (i.e., in the top 25% of sequences), below 0.01% frequency in the blood, and above 0.75 in the tumor (i.e., in the top 25% of sequences). Only the top 5 sequences are shown for simplicity, but up to 20 were found for each chain of the TCR. Similar analyses can be done in the future either using either single-cell sequencing as proposed earlier or sequencing the alpha and beta chains separately to predict tumor-specific TCRs.

PATIENT	SPECIMEN	DIAGNOSIS	PATHOLOGY	HLA
1 (C) *65 yr, F*	Tumor Infiltrating Lymphocytes (TILs) Lymph Node (LN) LN Post expansion	Invasive Ductal Carcinoma *Luminal A	LN+, DCIS, Stage 2b, ER+. PR+, HER2-,	A02:01 DRB1:12:02
2 (D) *50 yr, F*	TILs TILs Post expansion LN LN Post expansion	Invasive Ductal Carcinoma *Luminal A	LN+, Stage 2b, ER+, PR+, HER2-, Lymphovascular invasion	A02:01 DRB1:09:01
3 (E) *47 yr, F*	TILs LN	Invasive Ductal Carcinoma *Luminal A	LN-, Stage 2a, ER+. PR+, HER2-, lymphovascular invasion	A02:01
4 (197)	TILs Peripheral Blood (PB)	Invasive ductal carcinoma *Luminal B	LN-, Negative mets, DCIS, ER+, PR+, HER2+	A02:01 DRB1:13:01
5 (200) *62 yr, F*	TILs LN PB	Invasive ductal carcinoma *Luminal A	LN+, DCIS, ER+, PR+, HER2 -, Lymphovascular invasion	A02:01 DRB1:01:01
6 (211) *32 yr, F*	TILs PB	Invasive ductal carcinoma *Luminal A	LN-, DCIS, ER+, PR+, HER2-	A02:01 DRB1:04:01
7 (256) *79 yr, F*	TIL PB	Invasive ductal carcinoma *Basal-like	LN-, ER-, PR-, HER2-	A02:01 DRB1:13:04

Table 4. Summary of patient samples sequenced for TCR analysis. Patients C, D, and E were collected at City of Hope, patients 197, 200, 211, and 256 were collected at the University of Colorado. For publication, sample identifiers will be standardized (i.e. patient A-F) and the affiliation indicated in the methods section. Clinical information was deidentified prior to dissemination.

Sample	Total α reads	# Unique CDR3α sequences	Total β reads	# Unique CDR3β sequences	# Input CD8 cells
C1 TIL	551,950	980	2,952,753	1800	50,000
C1 LN	4,250,006	4201	3,656,661	4950	200,000
D1 TIL	2,085,766	2344	2,463,014	1444	38,000
D1 LN	1,564,348	4371	467,900	4102	1,240,000
E1 TIL	813,514	3330	1,512,421	5644	20,000
E1 LN	3,144,080	6746	1,460,805	5066	190,000
197 TIL	708,105	548	5,013,033	1922	<4,000
197 PB	4,323,463	36320	2,005,202	165848	1,000,000
200 TIL	963,489	1480	4,081,885	5430	30,000
200 LN	2,204,462	5727	2,026,775	802	37,500
200 PB	4,503,650	22521	3,118,472	13798	1,000,000
211 TIL	963,211	616	1,515,860	1957	16,000
211 PB	2,354,303	10487	2,875,636	37700	1,000,000
256 TIL	2,754,688	5193	1,903,965	3740	256,000
256 PB	4,751,007	38391	1,627,181	24748	1,000,000

Table 5. Sequencing summary for the samples listed in Table 4. Total CDR3 unique sequences encompass only sequences present 2 or more times.

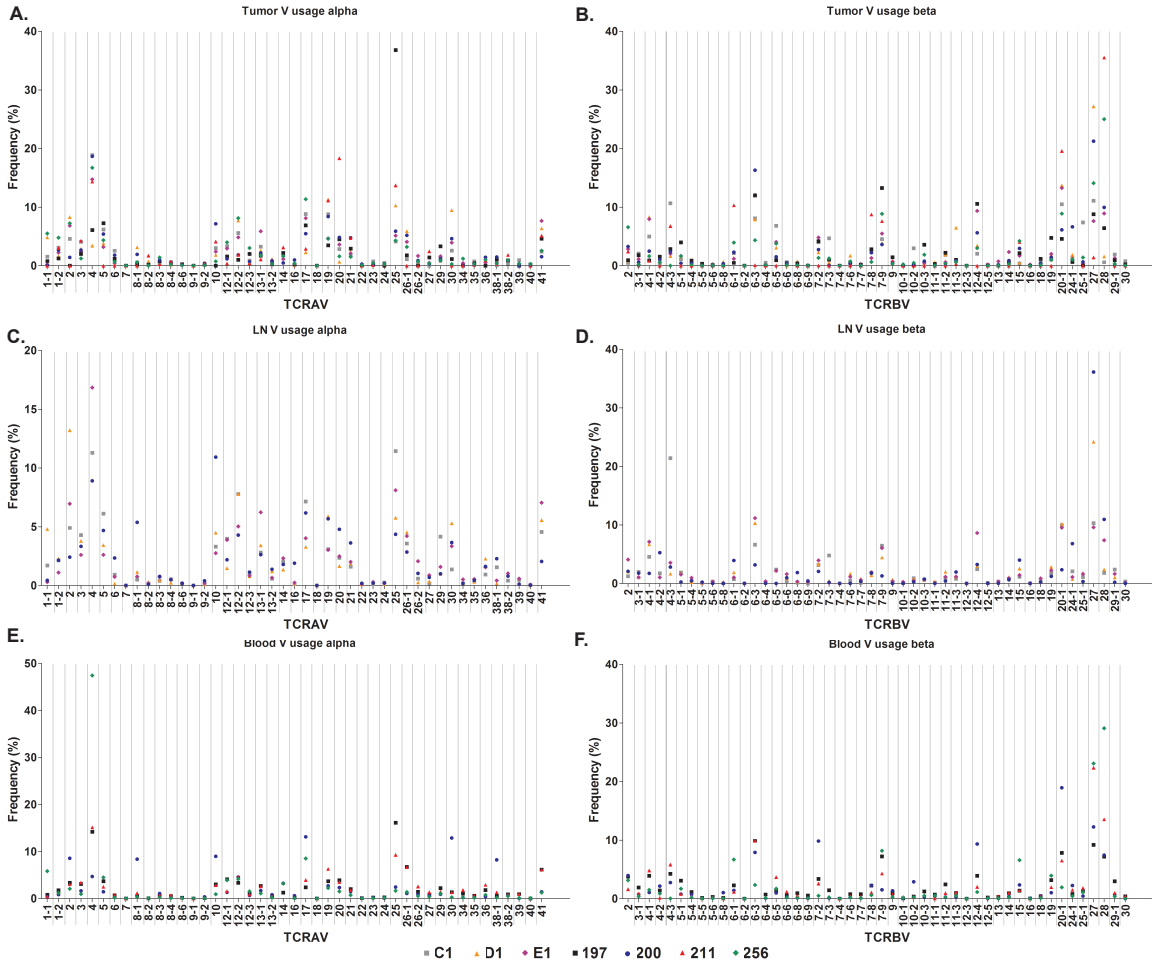


Figure 11. Variable region usage within the tumor, lymph node, and blood compartments is different from each other and among patients. For tumor samples (A & B), all seven patients are included. For lymph node samples (C & D) only patients C1, D1, E1, and 200 are included. For blood (E & F), only patients 197, 200, 211, and 256 are included.

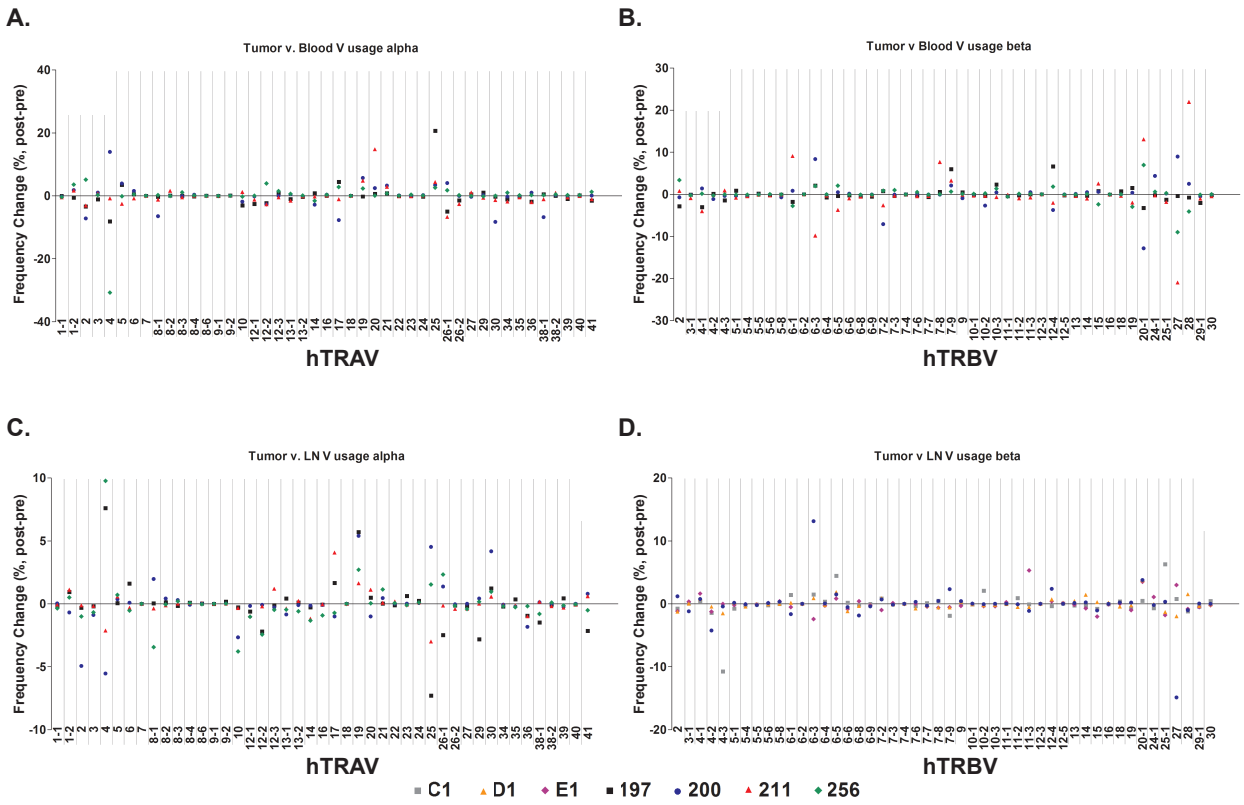


Figure 12. Frequency differences between the tumor and blood, and the tumor and lymph node reveal preferential V usage by TIL. For the tumor versus blood comparison (A & B), only patients 197, 200, 211, and 256 are included. For the tumor versus lymph node comparison (C & D), only patients C1, D1, E1, and 200 are included.

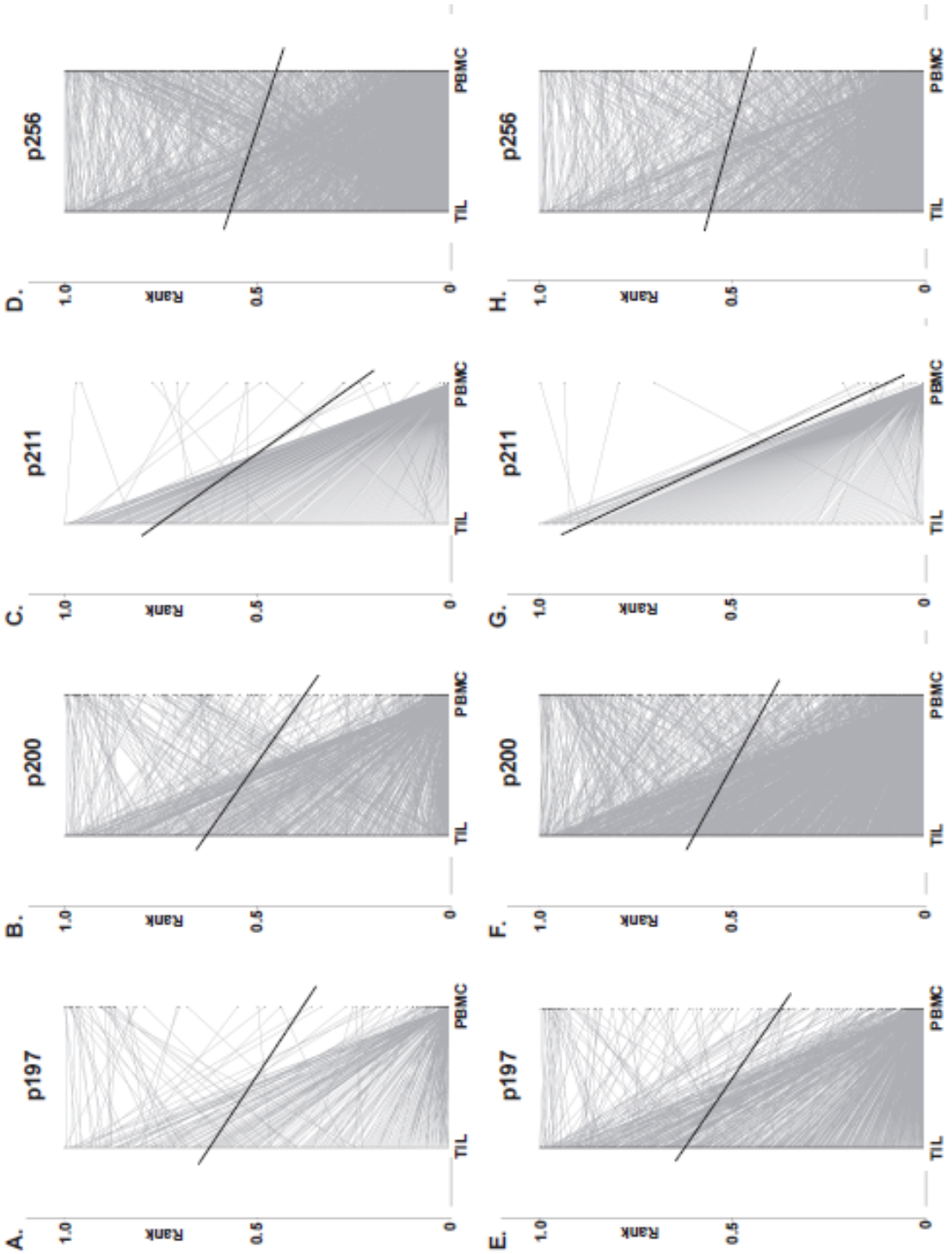


Figure 13. CDR3 differences between the tumor and blood provide information on which CDR3s are tumor specific. Unique CDR3 sequences were ranked and compared to the unique CDR3 sequences from the blood and the ranking in the blood plotted. For CDR3 alpha sequences (A-D) and CDR3 beta (E-H) the overall trend of change was plotted as a weighted average of the slopes and is shown as a black line. Only tumor derived CDR3 sequences are shown, blood CDR3 sequences which were not present in the tumor are not shown for simplicity.

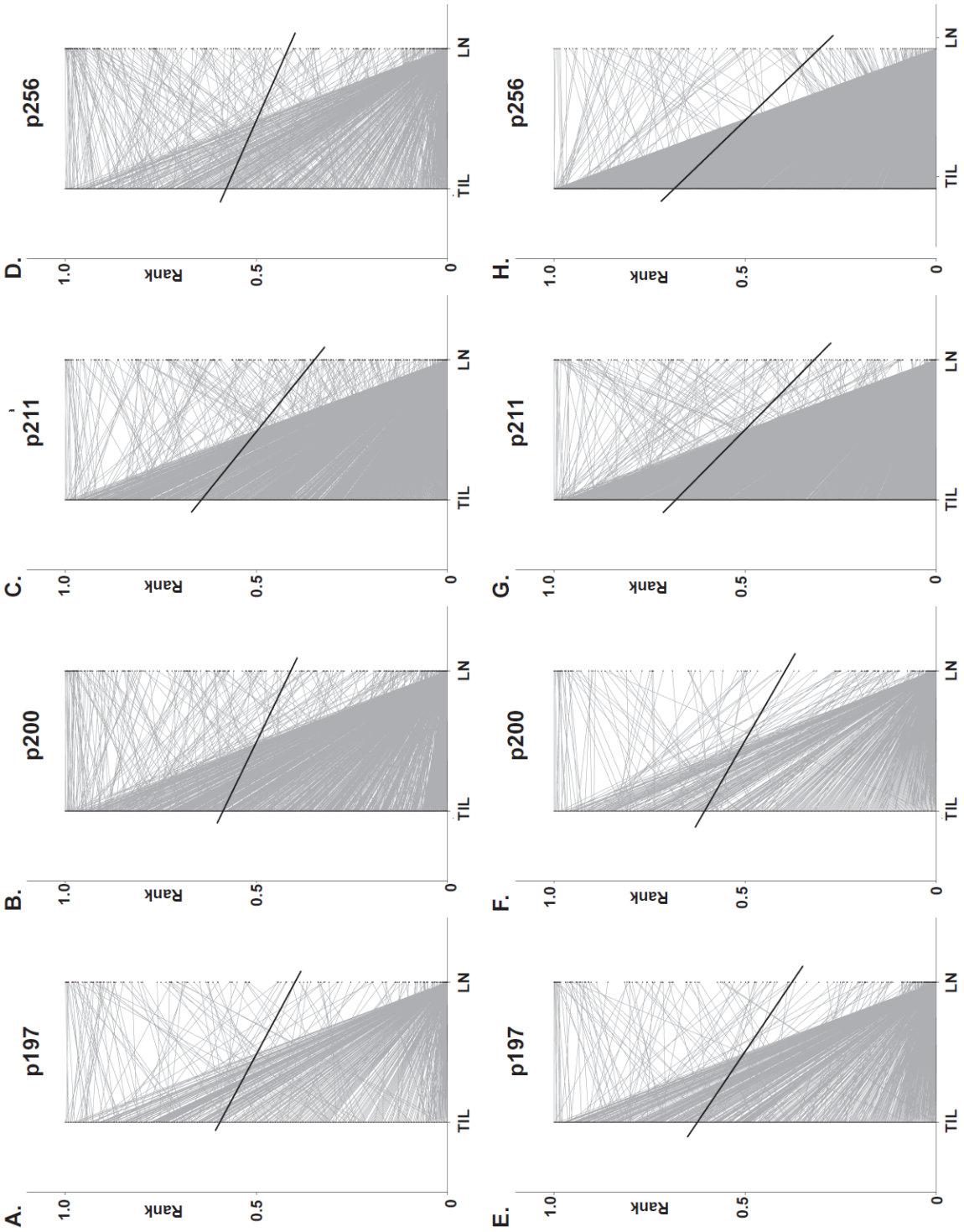


Figure 14. CDR3 identified in the tumor are more similar to the unique CDR3 sequences from the lymph node than from the blood. The alpha (A-D) and beta (E-H) CDR3 sequences found in the tumor were plotted according to rank and the corresponding rank within the lymph node was also plotted. Trend lines (black lines) indicate the similarity between the TIL and lymph node populations and are calculated by determining the weighted average of the slopes of the individual lines.

A.

Alpha CDR3_V_J sequence	TIL Count	TIL freq	TIL Rank	B Freq	Blood Rank	LN Freq	LN Rank
LVGDTLYSGTYKYI_TRAV4_TRAJ40	41193	4.982063941	1	0.005104386	0.205307263	0.862437183	0.974528302
ALSETDNQGGKLI_TRAV19_TRAJ23	10961	1.325671907	0.981288981	0.006794352	0.272346369	0.725580443	0.969811321
GTEGGSNYKLT_TRAV30_TRAJ53	10295	1.245122916	0.979209979	0.000931206	0.036312849	0.234487027	0.872641509
VVANFGNEKLT_TRAV10_TRAJ48	8608	1.041089661	0.968814969	0	0	0.524871751	0.960377358
AVWPGGKLI_TRAV21_TRAJ23	7998	0.967313558	0.962577963	0.002172813	0.086592179	0.269572911	0.898113208

B.

Beta CDR3_V_J sequence	TIL Count	TIL freq	TIL Rank	B Freq	B Rank	LN Freq	LN Rank
ASSLQNNQPQHhTRBV28hTRBJ1-5	63873	1.604366324	0.991126886	0.008564081	0.282548476	0.433763756	0.704453441
ATSEGQISTDTQYhTRBV24-1hTRBJ2-3	61948	1.556014044	0.990239574	0.019982855	0.541551247	1.049360605	0.846153846
ATSDGWRNQPQHhTRBV24-1hTRBJ1-5	16712	0.419773144	0.970718722	0.012535828	0.396121884	1.21500116	0.886639676
ASRTGSSYEQYhTRBV28hTRBJ2-7	16573	0.416281732	0.969831411	0	0	1.063239924	0.850202429
ASSVGVSNQPQHhTRBV4-3hTRBJ1-5	16008	0.402090024	0.968056788	0.002316853	0.076177285	1.19740667	0.882591093

Table 6. Selected CDR3 sequences from patient 211 which were high frequency in the tumor, infrequent in the blood, and frequent within the lymph node are candidates for tumor specific clones. Alpha (A) and beta (B) CDR3VJ sequences were manually screened and sorted based on the rank in the tumor. The rank and frequency (%) in all three compartments are shown.

Task 6. Generate MHC/peptide baculovirus libraries: months 1-40, Slansky

6a. HLA-A*0201
6b. HLA-DR*0401
6c. HLA allele most common in enrolled patients with basal tumors

6a. An HLA-A2 peptide library has been constructed following the methods outlined in last year's annual report. The peptide library sequence constraints are X(ILM)LXXXXX(LV), yielding a potential library size of $2.56x10^8$ possible peptide combinations. PCR amplicons were generated from the baculoviral DNA which encodes the library and sequenced on the HiSeq2500. Figure 15 shows the distribution of amino acids at each position as well as the expected amino acids at each position following analysis of ~150 million sequence reads.

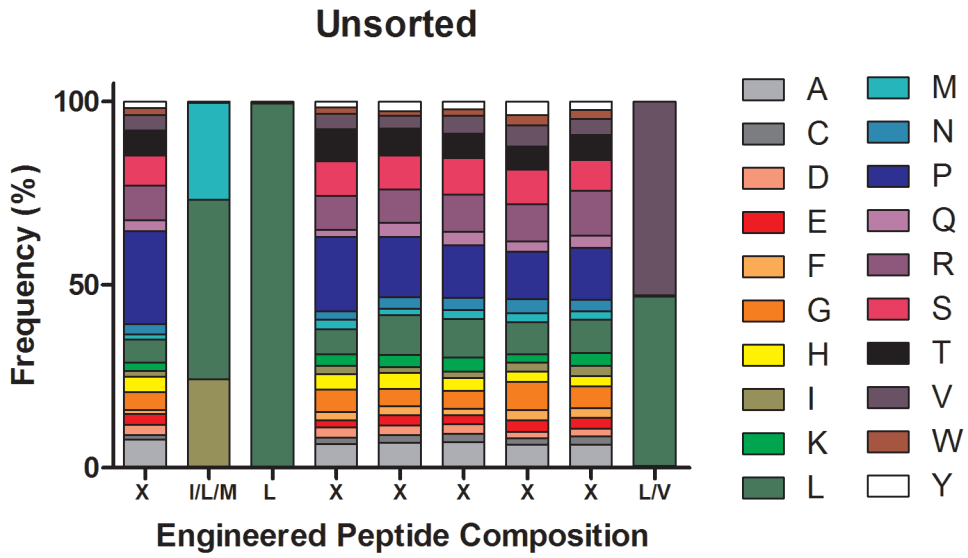


Figure 15. Sequencing of PCR amplicons of the peptide coding region of purified baculovirus DNA was performed to determine the amino acid distribution along the peptide. ~150 million DNA sequences encoding peptide sequence were translated. Unique peptide sequences were sorted and the frequency of a given amino acid at each position was determined. All 20 amino acids are represented at the randomized positions as shown by the colors. At the set positions, very small proportions were found of contaminating amino acids due to mutations either from the original peptide-encoding oligo, sequencing, or introduced by the viral polymerase during viral replication.

Recently, data obtained by OHSU and gathered from online sources (Figure 16) shows that perhaps our constraint of position 3 to leucine, which was done to decrease library size to workable levels, may have been too stringent. Additionally, position 2 appears to have more options than the initial engineered amino acids. Thus, we are in the process of building an alternate peptide library with the composition of X(LPAVMT)XXXXXX(LV). Isoleucine was excluded in the second library due to low frequency in Figure 16. Although the theoretical size of this library is very large ($1.54x10^{10}$) the odds of encountering any given

peptide combination are equal, meaning that no particular peptide should dominate any given library and that combining multiple library preps should cover all possible combinations.

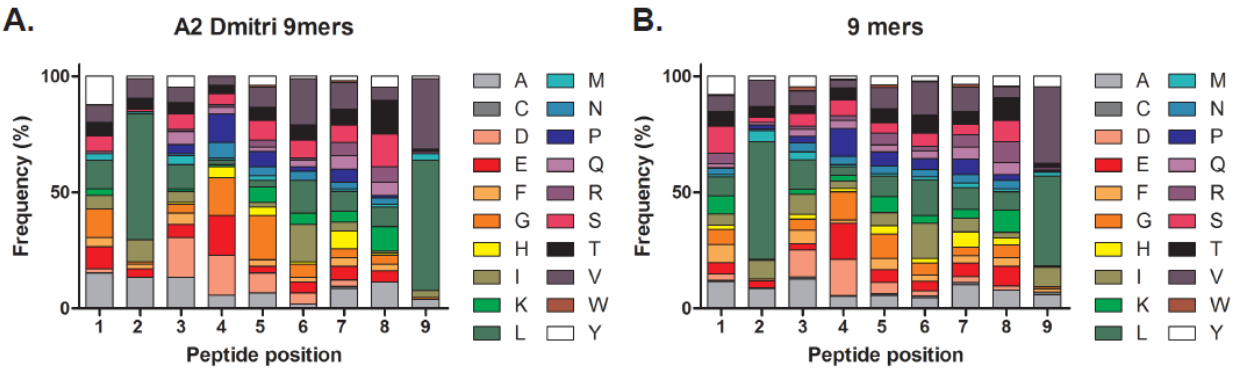


Figure 16: Peptides eluted from either HLA-A2+ breast cancer cells lines (done by Dmitri Rozanov, OHSU team) (A), or from an online peptide database (http://www.syfpeithi.de/, [4]*) (B), were compiled into a frequency distribution chart. The alternate peptide library proposed above is based on these findings.*

We have not yet addressed subsequent tasks.

KEY RESEARCH ACCOMPLISHMENTS:

- Obtained 4 HLA-A2+ patient samples from University of Colorado and 4 from the Lee lab City of Hope in the last 12 months.
- Developed an IFN-γ+ CD107a+ capture assay to identify activated T cells for future use for single cell sequencing and screening of HLA-peptide libraries.
- Determined that culturing T cells *in vitro* results in skewing of the repertoire.
- Began development of the single cell sequencing so that α and β chains of the TCR of one T cell can be determined at the same time.
- Generated a panel of cell lines that express commonly cited breast cancer antigens. We will soon determine if they also process and present tumor antigens. Currently we are collaborating with the Spellman lab to identify other relevant antigens from their RNAseq data.
- Generated a panel of positive control T cell clones specific for the commonly cited breast cancer antigens (above).
- Successfully generated first set of TCR transfectomas. We will soon test these against the commonly cited breast cancer antigens (2 bullets above).
- Performed deep sequencing on the TCRs from 7 HLA-A2+ samples and analyzed the results. Determined that the important T cells to analyze are present in the tumor and LN, but not frequently in the blood.
- Engineered an HLA-A2-peptide library.

REPORTABLE OUTCOMES:

None, however, manuscripts describing the repertoire skewing (Figures 4-7) and the TCR sequencing (Table 4-Figures 14) are in progress.

CONCLUSIONS:

This year we focused on identifying T cells from breast cancer patients that have the highest probability of being tumor-antigen specific. The studies that we performed suggest that we should not use T cells that have been expanded with CD3 and CD28 antibodies unless the same clonotypes can be identified *ex vivo*. Many studies have shown the skewing of the T cell repertoire when T cells are cultured with antigen. For example, the concentration of antigen is associated with the affinity of the T cells that are recovered (5). We showed this year that there is significant skewing of the repertoire with non-specific expansion. The skewing may be due to *in vitro* conditions such as the fetal calf serum or the plastic that the T cells are grown on (6). We concluded that antigen discovery of *ex vivo* T cells will give a more true representation of what the T cells in the tumor are recognizing than if we expand them in culture. Future experiments will require both *ex vivo* sequencing and expanding T cells in culture. However, we now understand that the T cells that we wish to identify cognate antigens and activating mimotopes must also be present *ex vivo*.

We have also proved the T cells that we will further analyze should be present in the tumor, and when possible from the tumor draining lymph nodes. The repertoire of these tissue resident T cells is different from those found in the blood. In Figure 13 we showed that the interesting T cells can be narrowed down by subtracting the TCRs identified in the blood from those in the tumor. The clonotypes most frequently identified represent T cells that have proliferated and clonally expanded in response to antigens in the tumor.

We also generated a set of cell lines that express the commonly cited breast cancer antigens that we can test these T cells against. As the Spellman lab determines other possible antigen candidates, we will add these to the panel. For example, we will determine if breast cancer patients make a *de novo* response to alternately spliced MMP11 gene identified by the Spellman lab.

This year we compiled the antigen panel, the positive-control T cells that recognize them, the protocols to generate TCR transfectomas, produced an HLA-A2 library, and identified T cells, for which we will identify the cognate antigen. Specifically, we want to use T cells that express IFN-γ and CD107a, can be found at high frequencies *ex vivo*, and are found in the tumor not the blood. We will single-cell sequence the α and β chains from these T cells; we are currently trouble shooting this protocol. Thus, this year we have optimized the procedures that are required for antigen and mimotope discovery.

References:

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